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(54) Title: PEPTIDES

(57) Abstract

The first group of peptides of the invention are known as Caerins. These peptides have the formula: W-Gly-Leu-X-Z, wherein W is hydrogen, C_{1-6} alkyl, C_{6-10} aryl, C_{7-16} aralkyl or C_{1-20} acyl; X is a peptide sequence comprising 20 to 23 amino acid residues; and Z is hydroxy, amino, C_{1-6} alkylamino, di-(C_{1-6} alkyl)-amino, C_{1-18} alkoxy or C_{7-18} aralkoxy. Generally, Caerins have a molecular weight of between 2300 and 2700. The second group of peptides of the invention are known as Caeridins. These peptides have the formula: W-Gly-Leu-Y-Z, wherein W is hydrogen, C_{1-6} alkyl, C_{6-10} aryl, C_{7-16} aralkyl or C_{1-20} acyl; Y is a peptide sequence comprising 8 to 13 amino acid residues; and Z is hydroxy, amino, C_{1-6} alkylamino, di-(C_{1-6} alkyl)-amino, C_{1-18} alkoxy or C_{7-18} aralkoxy. Generally, Caeridins have a molecular weight of between 1100 and 1600. Representative members of both groups of peptides may be isolated from the skin or glands of *Litoria splendida* and *Litoria caerulea*. Some of the present peptides have potent physiological activity. For example, one of the Caerins has potent antibiotic activity against a variety of bacteria. Some of the peptides also have anti-viral activity, and may have anti-fungal activity. The peptides may also be used for food preservation.

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PEPTIDES

BACKGROUND OF THE INVENTION

Amphibian skin is known to be a source of peptides, including some which are homologous to bioactive peptides of the mammalian gut and brain. In particular, a group of antimicrobrial peptides known as magainins has been isolated from the African clawed frog, Xenopus laevis (B.W. Gibson, L. Poulter, D.H. Williams and J.E. Maggio, J. Biol. Chem. 1986, 261, 5341; M.G. Giovannini, L. Poulter, B.W. Gibson and D.H. Williams, Biochem. J. 1987, 243, 113; M. Zasloff, Proc. Natl. Acad. Sci. U.S.A. 1987, 84, 5449; M Zasloff, B. Martin and H-C. Chen, Proc. Natl. Acad. Sci. U.S.A. 1988, 85, 910; A.S. Terry, L. Poulter, D.H. Williams, J.C. Nutwing, M.G. Giovannini, C.H. Moore and B.W. Gibson, J. Biol. Chem. 1988, 263, 5745; United States Department of Health and Human Services, U.S. Patent Application Serial No. 21,493 of 15th August 1987). Magainin II has the formula:

H_2N-Gly-Ile-Gly-Lys-Phe-Leu-His-Ser-Ala-Lys-Lys-
Phe-Gly-Lys-Ala-Phe-Val-Gly-Glu-Ile-Met-Asn-Ser-OH.

It has now been found that novel biologically active peptides occur in the skin and glands of two Australian species of frog: i) Litoria splendida, the Magnificent Tree Frog. This species was discovered by a group of zoologists from the Universities of Adelaide and Melbourne in 1977 and named by them (M.J. Tyler, M. Davies and A.A. Martin, Trans.R. Soc. S. Aust. 1977, 101, 133), and ii) Litoria caerulea.

SUMMARY OF THE INVENTION

According to one aspect of the invention, there is provided a group of peptides, known as Caerins, which generally have a molecular weight of between 2300 and 2700. Representative members of this group of peptides may be isolated from the skin or glands of Litoria splendida and Litoria caerulea. Similarly, there is provided a second group

of peptides, known as Caeridins, which generally have a molecular weight between 1100 and 1600. Representative members of this second group of peptides may also be isolated from Litoria splendida and Litoria caerulea.

According to a second aspect of the invention, there is provided a method for the preparation of these peptides by (a) extraction from frog skin and/or glands; (b) conventional methods of peptide synthesis; or (c) recombinant DNA technology.

According to a third aspect of the invention, there are provided methods for the treatment of humans and animals which comprise administration of at least one of the peptides of the invention.

According to a fourth aspect of the invention, there are provided pharmaceutical or veterinary compositions comprising at least one of the peptides of the invention.

DETAILED DESCRIPTION

A) The first group of peptides of the invention are known as Caerins. These peptides have the formula:

W-Gly-Leu-X-Z,

wherein W is hydrogen, C_{1-6} alkyl, C_{6-10} aryl, C_{7-16} aralkyl or C_{1-20} acyl; X is a peptide sequence comprising 20 to 23 amino acid residues; and Z is hydroxy, amino, C_{1-6} alkylamino, di-(C_{1-6} alkyl)-amino, C_{1-18} alkoxy or C_{7-18} aralkoxy.

Preferably, W is hydrogen and Z is amino or hydroxy.

Generally, Caerins have a molecular weight of between 2300 and 2700.

Representative members of this group of peptides may be isolated from the skin or glands of Litoria splendida and Litoria caerulea.

In particular, Caerin 1 has the structure:
H-Gly-Leu-Leu-Ser-Val-Leu-Gly-Ser-Val-Ala-Lys-His-Val-Leu-Pro-His-Val-Val-Pro-Val-Ile-Ala-Glu-His-Leu-NH_2.

Caerin 2 has the structure:
H-Gly-Leu-Val-Ser-Ser-Ile-Gly-Arg-Ala-Leu-Gly-Gly-Leu-Leu-Ala-Asp-Val-Val-Lys-Ser-Lys-Gly-Gln-Pro-Ala-OH.

Caerin 3 has the structure:
H-Gly-Leu-Trp-Gln-Lys-Ile-Lys-Asp-Lys-Ala-Ser-Glu-Leu-Val-Ser-Gly-Ile-Val-Glu-Gly-Val-Lys-NH_2.

Caerin 4 has the structure:
H-Gly-Leu-Trp-Gln-Lys-Ile-Lys-Ser-Ala-Ala-Gly-Asp-Leu-Ala-Ser-Gly-Ile-Val-Glu-Gly-Ile-Lys-Ser-NH_2.

Other peptides have structures related to Caerins 1-4.

B) The second group of peptides of the invention are known as Caeridins. These peptides have the formula:

W-Gly-Leu-Y-Z,
wherein W is hydrogen, C_{1-6} alkyl, C_{6-10} aryl, C_{7-16} aralkyl or C_{1-20} acyl; Y is a peptide sequence comprising 8 to 13 amino acid residues; and Z is hydroxy, amino, C_{1-6} alkylamino, di-(C_{1-6} alkyl)-amino, C_{1-18} alkoxy or C_{7-18} aralkoxy.

Preferably, W is hydrogen and Z is amino or hydroxy.

Generally, Caeridins have a molecular weight of between 1100 and 1600.

Representative members of this group of peptides may also be isolated from the skin or glands of Litoria splendida and Litoria caerulea.

In particular, Caeridin 1 has the structure:
H-Gly-Leu-Leu-Asp-Gly-Leu-Leu-Gly-Thr-Leu-NH_2.

Caeridin 2 has the structure:
H-Gly-Leu-Leu-Gly-Met-Val-Gly-Ser-Leu-Leu-Gly-Gly-Leu-Gly-Leu-NH_2.

Other peptides have structures related to Caeridins 1 and 2.

C) Peptides of both groups A) and B) were isolated from the frog Litoria splendida or Litoria caerulea by methanol/water extraction of skin and/or glandular material, followed by HPLC separation of the various peptides present in that extract. The structures of the peptides were then determined, e.g. by a combination of fast atom bombardment mass spectrometry and degradative methods (Edman degradation, enzymic digestion).

The peptides can also be prepared by means of conventional synthetic methods. For this purpose, the amino acids are provided with protecting groups, as required, and are coupled in the correct order, or smaller peptides are combined to form bigger units. After synthesis, any protecting groups present are removed in conventional manner.

Peptides are usually prepared by:

a) condensing an amino acid or peptide having a protected α-amino group and an activated terminal carboxyl group with an amino acid or peptide, the α-amino group of which is free;

b) condensing an amino acid or peptide having an activated α-amino group and a protected carboxyl group with an amino acid or peptide having a free terminal carboxyl group and a protected α-amino group; or

c) condensing an amino acid or peptide having a free carboxyl and a protected α-amino group with an amino acid or peptide having a free α-amino group and a protected carboxyl group.

Activation of the carboxyl group can take place, for example, by converting the carboxyl group into an acid halide, an azide, anhydride or imidazolide, or into an activated ester such as the cyanomethyl ester or p-nitro-phenyl ester.

The amino group can be activated by, for example, converting the amino group into a phosphate amide.

Commonly-used methods for the condensation of amino acids or peptides are: The carbodiimide method, the azide method, the anhydride method and the activated ester method described in, for example, "THE PEPTIDES" Volume 1, 1965 (Academic Press) by E. Schroder and K. Lubke. Furthermore the so-called "solid phase" method of Merrifield, described in J. Am. Chem. Soc. 85, 2149 (1963), can be used for the manufacture of the present peptides.

The free functional groups in the amino acid or peptide, which should not participate in the condensation reaction, are protected effectively by so-called protecting groups, which can be removed again quite easily by hydrolysis or reduction. Thus, for example, the carboxyl group can be protected effectively by, for example, esterification with methanol, ethanol, tertiary butanol, benzyl alcohol or p-nitrobenzyl alcohol or by forming an amide. The latter group, however, is very difficult to remove so that it is to be preferred to use it only to protect the terminal hydroxyl group in the ultimate

peptide. The N-protecting groups are generally acyl groups, for example, an acyl group derived from an aliphatic, aromatic, araliphatic or heterocyclic acid such as acetic acid, chloro-acetic acid, butyric acid, benzoic acid, phenyl-acetic acid or pyridine-carboxylic acid, or an acyl group derived from carbonic acid such as ethoxy-carbonyl, benzyloxy-carbonyl, t-butoxy-carbonyl or p-methoxy-benzyloxy-carbonyl, or an acyl group derived from a sulphonic acid such as benzenesulfonyl or p-toluene-sulfonyl, but other groups, too, can be used, such as substituted or unsubstituted aryl or aralkyl groups, for example, benzyl and triphenyl-methyl.

The guanidine group of arginine should preferably be protected by a nitro group, while the imino group of histidine should preferably be protected by a benzyl or trityl group. Generally, it is preferred to use a tertiary butylester to protect the carboxyl group and a butoxy-carbonyl, benzyloxy-carbonyl or tosyl group to protect the amino group.

The protecting groups can be split off by various conventional methods, dependent upon the nature of the protecting group, for example: by means of trifluoro-acetic acid or by mild reduction, for example with hydrogen and a catalyst such as palladium, or with HBr in glacial acetic acid.

Peptides wherein the N-terminal amino group is alkylated, arylated, aralkylated or acylated are prepared by conventional methods. C-Terminal amides and esters are prepared by reaction with an appropriate amine or alcohol, respectively, or an activated derivative thereof.

Recombinant DNA techniques may also be used to synthesize the present peptides. A mRNA sequence encoding a Caerin or a Caeridin is isolated from the frog, the complementary cDNA sequence is produced therefrom, and that cDNA sequence is then expressed in a suitable expression system. Alternatively, a

synthetic DNA sequence encoding a Caerin or a Caeridin may be used.

Some of the present peptides have potent physiological activity. For example, Caerin 1 has potent antibiotic activity against a variety of bacteria. Some of the peptides also have anti-viral activity, and may have anti-fungal activity.

The invention thus relates to a method of treating humans and animals which comprises administration of at least one of these peptides, either by itself or in the form of a pharmaceutical or veterinary composition.

The peptides may also be used for food preservation.

The invention also relates to pharmaceutical or veterinary compositions which comprise at least one of these peptides. The peptides may be used either as such or (preferably) in combination with suitable carriers, adjuvants or auxiliary substances. The compositions may be in the form of tablets, dragees, capsules, suppositories, syrups, emulsions, suspensions or solutions.

Suitable excipients are solvents, gelling agents, antioxidants, dispersing agents, emulsifiers, anti-foaming agents, flavouring and colouring agents, preservatives and solubilizing agents.

The compositions may be administered orally, parenterally or rectally.

Suitable dosage unit compositions for oral administration may be prepared by mixing the active ingredient with a solid pulverulent carrier such as lactose, sucrose, sorbitol, mannitol, a starch (e.g. potato starch, corn starch or amylopectin), a cellulose derivative or gelatine, and a lubricant such as magnesium stearate, calcium stearate or a

polyethylene glycol wax. The mixture is then compressed to form tablets. Coated tablets can be prepared by coating such tablets with a concentrated sugar solution which may contain e.g. gum arabic, gelatine, talcum or titanium dioxide, or by coating such tablets with a lacquer dissolved in a readily volatile organic solvent.

Soft gelatine capsules can be prepared by enclosing the active ingredient, mixed with a vegetable oil, in a soft gelatine shell. Hard gelatine capsules may contain the active ingredient in admixture with a solid pulverulent carrier such as lactose, sucrose, sorbitol, mannitol, a starch (e.g. potato starch, corn starch or amylopectin), a cellulose derivative or gelatine.

Dosage unit preparations for rectal administration can be prepared in the form of suppositories comprising the active ingredient in admixture with a fatty base, or in the form of gelatine capsules comprising the active ingredient in admixture with a vegetable oil or paraffin oil.

Liquid preparations for oral administration can be in the form of syrups, solutions, emulsions, or suspensions of the active ingredient. Sugar, flavouring agents and colouring agents may be added, and the solvent may be e.g. ethanol, water, glycerol, propylene glycol or a mixture thereof.

Compositions for parenteral administration by injection can be prepared as an aqueous solution of the active ingredient. Such solutions may also contain stabilizing agents and/or buffering agents, and may conveniently be provided in suitable dosage unit ampoules.

The foregoing describes particular embodiments of the present invention. It will be obvious to those skilled in the art that modifications and variations can be made, without departing from the inventive concept, as defined by the accompanying claims.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A peptide having the formula:
W-Gly-Leu-X-Z,
wherein W is hydrogen, C_{1-6} alkyl, C_{6-10} aryl, C_{7-16} aralkyl or C_{1-20} acyl; X is a peptide sequence comprising 20 to 23 amino acid residues; and Z is hydroxy, amino, C_{1-6} alkylamino, di-(C_{1-6} alkyl)-amino, C_{1-18} alkoxy or C_{7-18} aralkoxy.

2. A peptide according to claim 1, wherein W is hydrogen.

3. A peptide according to claim 1 or claim 2, wherein Z is amino or hydroxy.

4. A peptide according to any one of claims 1 to 3, having a molecular weight of between 2300 and 2700.

5. A peptide having the structure:
H-Gly-Leu-Leu-Ser-Val-Leu-Gly-Ser-Val-Ala-Lys-His-Val-Leu-Pro-His-Val-Val-Pro-Val-Ile-Ala-Glu-His-Leu-NH_2.

6. A peptide having the structure:
H-Gly-Leu-Val-Ser-Ser-Ile-Gly-Arg-Ala-Leu-Gly-Gly-Leu-Leu-Ala-Asp-Val-Val-Lys-Ser-Lys-Gly-Gln-Pro-Ala-OH.

7. A peptide having the structure:
H-Gly-Leu-Trp-Gln-Lys-Ile-Lys-Asp-Lys-Ala-Ser-Glu-Leu-Val-Ser-Gly-Ile-Val-Glu-Gly-Val-Lys-NH_2.

8. A peptide having the structure:
H-Gly-Leu-Trp-Gln-Lys-Ile-Lys-Ser-Ala-Ala-Gly-Asp-Leu-Ala-Ser-Gly-Ile-Val-Glu-Gly-Ile-Lys-Ser-NH_2.

9. A peptide having the formula:
W-Gly-Leu-Y-Z,
wherein W is hydrogen, C_{1-6} alkyl, C_{6-10} aryl, C_{7-16} aralkyl or C_{1-20} acyl; Y is a peptide sequence comprising 8 to 13 amino acid residues; and Z is hydroxy, amino, C_{1-6} alkylamino, di-(C_{1-6} alkyl)-amino, C_{1-18} alkoxy or C_{7-18} aralkoxy.

10. A peptide according to claim 9, wherein W is hydrogen.

11. A peptide according to claim 9 or claim 10, wherein Z is amino or hydroxy.

12. A peptide according to any one of claims 9 to 11, having a molecular weight of between 1100 and 1600.

13. A peptide having the structure:
H-Gly-Leu-Leu-Asp-Gly-Leu-Leu-Gly-Thr-Leu-NH_2.

14. A peptide having the structure:
H-Gly-Leu-Leu-Gly-Met-Val-Gly-Ser-Leu-Leu-Gly-Gly-Leu-Gly-Leu-NH_2.

15. A method for preparing a peptide according to any one of claims 1 to 14, comprising extracting said peptide from frog skin and/or glands.

16. A method according to claim 15, wherein said frog is Litoria splendida or Litoria caerulea.

17. A method according to claim 15 or claim 16, comprising:
(a) methanol/water extraction of skin and/or glandular material; followed by
(b) HPLC separation of the various peptides present in the extract from step (a); and
(c) isolation of the desired peptide.

18. A method for preparing a peptide according to any one of claims 1 to 14, comprising coupling, in the correct order, the constituent amino acid or peptide sub units.

19. A method for preparing a peptide according to any one of claims 1 to 14, by recombinant DNA techniques.

20. A method according to claim 19, wherein a mRNA sequence encoding the peptide is isolated from a frog, the complementary cDNA sequence is produced therefrom, and that cDNA sequence is then expressed in an appropriate expression system.

21. A method according to claim 20, wherein said frog is Litoria splendida or Litoria caerula.

22. A method according to claim 19, wherein a DNA sequence encoding the peptide is synthesized, and that DNA sequence is then expressed in an appropriate expression system.

23. A pharmaceutical or veterinary composition comprising a peptide according to any one of claims 1 to 14, together with an appropriate carrier therefor.

24. A pharmaceutical or veterinary composition having antibiotic, anti-bacterial, anti-viral or anti-fungal activity, comprising a peptide according to any one of claims 1 to 14, together with an appropriate carrier therefor.

25. A food-preserving composition comprising a peptide according to any one of claims 1 to 14, together with an appropriate carrier therefor.

26. A method for treating or preventing bacterial, viral and fungal diseases in humans or animals, comprising administration to said human or animal of a peptide according to any one of claims 1 to 14 or a composition according to claim 23 or claim 24.

27. A method for preserving food, comprising adding thereto a peptide according to any one of claims 1 to 14 or a composition according to claim 25.

28. A peptide according to any one of claims 1 to 14, substantially as described herein.

29. A method for preparing a peptide according to any one of claims 15 to 22, substantially as described herein.

30. A pharmaceutical or veterinary composition according to claim 23 or claim 24, substantially as described herein.

31. A food-preserving composition according to claim 25, substantially as described herein.

32. A method for treating or preventing bacterial, viral and fungal diseases in humans or animals according to claim 26, substantially as described herein.

33. A method for preserving food according to claim 27, substantially as described herein.

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent classification (IPC) or to both National Classification and IPC

Int. Cl.[5] C07K, 7/10, 7/06, 7/08, //A61K, 37/02, A23L, 3/3526

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	C07K 7/10, 7/06, 7/08

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU: IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category*	Citation of Document, [11] with indication, where appropriate of the relevant passages [12]	Relevant to Claim No [13]
P,X	WO,A,91/06558 (GESELLSCHAFT FUR BIOTECHNOLOGISHE FORSCHUNG MBH) 16 May 1991 (16.05.91), see figure 2A.	9

* Special categories of cited documents : [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18 May 1992	29 May 1992 (29.05.92)
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AUSTRALIAN PATENT OFFICE	NORMAN BLOM